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(54) Title: ANTIMICROBIAL PEPTIDES (57) Abstract The invention relates to peptides with antimicrobial activity, consisting of an amino acid chain which contains a domain of 10 to 25 amino acids, wherein the majority of the amino acids of the one half of the domain are positively charged amino acids and the majority of the amino acids of the other half of the domain are uncharged amino acids.		

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ANTIMICROBIAL PEPTIDES

The present invention relates to new peptides with an antimicrobial activity. The antimicrobial activity is particularly aimed at bacteria, fungi and yeasts.

The use of the known antibiotics is in an increasing number of cases no longer sufficient for the treatment of infections. Many bacteria strains have developed resistance to the known classes of antibiotic and in the last thirty years no new classes of antibiotic have been discovered. In view of the above, a new class of antimicrobial agents is very desirable. Alkaline peptides and proteins are found in saliva which have a bactericidal and fungicidal activity in vitro. Histatins form a known family of such salivary peptides. However, in order to be clinically applicable as well it is desirable that the antimicrobial activity is even higher. A higher activity compensates the proteolytic degradation of the agent which always occurs to a greater or lesser degree. Furthermore, a reduced proteolytic degradation relative to the naturally occurring peptides is desirable. Finally, from an economic point of view in respect of the production of the peptides, it is recommended that antimicrobial agents are relatively small.

It is the object of the present invention to provide new antibacterial and antifungal agents which do not have the above stated drawbacks and which comply as far as possible with the recommended requirements.

This is achieved with the invention by peptides consisting of an amino acid chain which contains a domain of 10 to 25 amino acids, wherein the majority of the amino acids of the one half of the domain are positively charged amino acids and the majority of the other half of the domain are uncharged amino acids.

The structure of these peptides has a number of variations. Firstly, the domain can form an α -helix, of which at least a majority of the positions 1, 2, 5, 6, 9 (12, 13, 16, 19, 20, 23 and 24) contains a positively charged amino acid, position 8 is a positive or an uncharged amino acid and at least a majority of the positions 3, 4, 7, 10, (11, 14, 15, 17, 18, 21, 22, 25) contains an uncharged amino acid. These peptides have a lateral amphipathicity, i.e. a maximum hydrophobic moment at 100°. Stated simply, these peptides are hydrophobic on the left side and hydrophilic on the right side or vice versa. These peptides are referred to herein as "type I".

The domain can further form an α -helix, of which at least a majority of the positions 1, 2, 5, 6, 9 (12, 13, 16, 19, 20, 23 and 24) contains an uncharged amino acid, position 8 is a positive or an uncharged amino acid and at least a majority of the positions 3, 4, 7, 10, (11, 14, 15, 17, 18, 21, 22, 25) contains a positively charged amino acid. These peptides have a lateral amphipathicity, i.e. a maximum hydrophobic moment at 100°. Stated simply, these peptides are hydrophobic on the right side and hydrophilic on the left side or vice versa. These peptides are designated "type II" herein and are in principle mirror-symmetrical to type I peptides.

In addition, the domain can form an α -helix, wherein at least a majority of the positions 1 to 6 (or 7 or 8 or 9 or 10 or 11 or 12) contains an uncharged amino acid and a positively charged amino acid is found at position 7 (or 8 or 9 or 10 or 11 or 12 or 13) to 25. These peptides have a longitudinal amphipathicity, i.e. a minimum hydrophobic moment at 100°. These peptides are hydrophobic on their "top" and hydrophilic on their "bottom". Such peptides are designated "type III".

Conversely, the domain can form an α -helix, wherein at least a majority of the positions 1 to 6 (or 7 or 8

or 9 or 10 or 11 or 12) contains a positively charged amino acid and an uncharged amino acid is found at position 7 (or 8 or 9 or 10 or 11 or 12 or 13) to 25. These peptides likewise have a longitudinal

5 amphipathicity and therefore a minimum hydrophobic moment at 100° . These peptides are hydrophobic on their "bottom" and hydrophilic on their "top". Such peptides are designated "type IV".

Finally, the domain can form a so-called β -strand
10 and contain a positively charged amino acid on at least a majority of the positions 1, 3, 5, 7, 9 (11, 13, 15, 17, 19, 21, 23 and 25) and an uncharged amino acid on at least a majority of the positions 2, 4, 6, 8, 10, (12, 14, 16, 18, 20, 22, 24). Such a β -strand is laterally
15 amphipathic and has a maximum hydrophobic moment at 180° . The β -strand structure is flatter than the α -helix and, stated simply, is hydrophobic on the left and hydrophilic on the right or vice versa. These are "type V" peptides.

20 The positively charged amino acids are preferably chosen from the group consisting of ornithine (O), lysine (K), arginine (R) and histidine (H), while the uncharged amino acids are preferably chosen from the group consisting of the aliphatic amino acids glycine
25 (G), alanine (A), valine (V), leucine (L), isoleucine (I), the amino acids with a dipolar side chain methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), the amino acids with an aromatic side chain phenylalanine (F), tyrosine (Y), tryptophan
30 (W). Amino acids on the border between hydrophilic and hydrophobic can be chosen from both groups or from the remaining amino acids.

Hardly any difference in activity can in principle be detected when one of the positive amino acids and/or
35 one of the uncharged amino acids is replaced by a random amino acid. The majority of the positively charged amino acids is therefore preferably the total number of

positively charged amino acids minus 1 and the majority of the uncharged amino acids is preferably the total number of uncharged amino acids minus 1.

The domain can be a part of a larger peptide but
5 can itself also make up the entire peptide. When the domain forms part of a larger peptide, the C-terminal and/or N-terminal amino acids which are then additionally present can be random amino acids.

The following peptides of the type I are
10 particularly recommended:

	KRLFKELKFSLRKY	(peptide 3)
	KRLFKELLFSLRKY	(peptide 4)
	KRLFKELKKSLRKY	(peptide 5)
	KRLFKELLKSLRKY	(peptide 6)
15	OOLFOELOOSLOOY	(peptide 7)
	OOLFOELLOSLOOY	(peptide 8)
	KRLFKKLKFSLRKY	(peptide 9)
	KRLFKKLLFSLRKY	(peptide 10)

A preferred peptide of the type III has the
20 following amino acid sequence:

LLLFLKKRKKRKY (peptide 11)

The peptides according to the invention can also contain further modifications. These modifications are for instance an N-terminal amide ring, for instance with
25 acetic acid anhydride, or an alternative cleavage of the synthesis resin by which the C-terminus is modified. For this latter a replacement of the C-terminal carboxylic acid group by an amide, ester, ketone, aldehyde or alcohol group can be envisaged. Peptides with such a
30 modification are for instance:

KRLFKELKFSLRKY-amide (peptide 12)

KRLFKELLFSLRKY-amide (peptide 13)

In addition to single peptides, oligomers can also be made. These are preferably linear oligomers of the
35 peptides according to the invention. The coupling can be head-to-head and tail-to-tail as well as head-to-tail, either by direct synthesis or by post-synthetic

enzymatic coupling. For a trans-membrane pore formation a minimum peptide length is required. Oligomers of the peptides according to the invention are double length and thereby better able in principle to span the whole phospholipid double layer of the bacterial cell membrane at one time. The activity of the peptide could hereby improve even further. In addition, extension of the peptides provides stabilisation of the helix conformation. A spacer must usually be inserted. In direct synthesis of head-to-tail coupled oligomers a spacer can be inserted to size by the use of a chain of unnatural amino acids of the correct length, for instance β -alanine, γ -amino butyric acid, ϵ -amino caproic acid, etc. Heterodifunctional coupling reagents, such as are commercially available for coupling peptide antigens to carrier proteins (for instance 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *m*-maleimidobenzoyl)-*N*-hydroxysuccinimide ester (MBS), *N*-succinimidyl 3-[pyridyldithio]propionate (SPDD) etc.) are used to make linear oligomers with an inserted spacer. For head-to-head and tail-to-tail couplings can be used trivalent amino acids such as asparagine acid (D), glutamine acid (E), ornithine (O), lysine (K), serine (S), cysteine. Such oligomers are for instance:

KRKFHEKHHSHRGYC-CYGRHSHHKEHFKRK (peptide 14)
 YGRHSHHKEHFKRKC-CKRKFHEKHHSHRGY (peptide 15)
 $^{\alpha}\text{N}, ^{\epsilon}\text{N}-(\text{KRKFHEKHHSHRGY})_2\text{K-amide}$ (peptide 16)
 $^{\alpha}\text{N}, ^{\epsilon}\text{N}-(\text{KRLFKEKLFSLRKY})_2\text{K-amide}$ (peptide 17)
 $^{\alpha}\text{N}, ^{\epsilon}\text{N}-(\text{KRLFKKLKFSLRKY})_2\text{K-amide}$ (peptide 18)

Peptides 14 and 15 are obtained by synthesis of peptide 2 with an additional C-terminal respectively N-terminal cysteine, whereafter the oligomer is obtained by air oxidation. Peptides 16, 17 and 18 are obtained by making use of the Multiple Antigenic Peptide (MAP) strategy, wherein a lysine having on both the α - and on the ϵ -amino group an Fmoc protection was used as first amino acid on the synthesis resin, whereby two identical amino

acid chains (peptides 2, 3 and 9) were synthesized simultaneously on one lysine molecule.

The peptides described herein have no or hardly any haemolytic activity in physiological buffers such as PBS
5 (phosphate-buffered saline solution). A low activity against erythrocytes of human origin is an indication of low toxicity. This selectivity is essential for the use of these peptides as antibiotics.

The peptides according to the invention can be used
10 in or as an antibacterial agent, in or as an antifungal agent and in or as an agent against infection by yeasts. Their activity will be further illustrated in the accompanying examples.

The invention therefore further relates to the
15 peptides for use as antibacterial agent, for use as antifungal agent and for use as anti-mycotic agent.

Also part of the invention is the use of the peptides for the manufacture of a medicine for the treatment of bacterial infections and for the
20 manufacture of a medicine for the treatment of fungal infections and/or infection by yeasts.

The peptides according to the invention can be used in different pharmaceutical forms of administration. Particularly recommended are spray, ointment, gel and
25 lozenges. These forms of administration can be used to control yeasts, such as Candida, bacteria in the oral cavity, on the skin, in cattle or in food, and fungi.

The invention is further illustrated in the accompanying examples, which are only given by way of
30 illustration and not to limit the invention in any way whatever.

EXAMPLES**EXAMPLE 1****Peptide synthesis**

Peptides according to the invention were chemically synthesized as described by Van 't Hof et al. (1991) and Helmerhorst et al. (1997). Peptides were synthesized using the T-bag method, which was adapted for 9-fluorenylmethoxycarbonyl ((Fmoc) chemistry). p-Benzylloxybenzyl alcohol resins to which the first N-Fmoc protecting amino acids were already coupled, were arranged in the T-bags. The coupling reactions were performed in N,N-dimethyl formamide. After completion of the amino acid chain it was cleaved from the resin and the side chain protection groups were simultaneously removed with a mixture of 5% thioanisole, 5% phenol, 5% water and 85% trifluoroacetic acid. Purity analyses were performed by reversed-phase HPLC and showed one single peak with only few contaminants (less than 5%).

All peptides were dissolved in 10 mM potassium phosphate buffer (PPB), pH 7.0, to a concentration of 2 mg/ml and stored at -80°C. The final pH of the stock solution was 6.0. The exact peptide concentrations which were used in the antibacterial assay were determined by amino acid analysis.

Table 1 gives an overview of the peptides 2 to 13 which were made in this manner. Peptides 1 and 2 from this table show respectively the histatin 5 and the C-terminal part thereof. Amino acids in bold type are changes relative to peptide 2.

30

EXAMPLE 2**Antibacterial activity against monocultures in vitro**

Monocultures of the bacteria Streptococcus mutans (R9), Streptococcus sanguis (SB 179), Streptococcus salivarius (SS 196), Actinomyces naeslundii (WVU 627), Fusobacterium nucleatum (ATCC 10953), Prevotella

intermedia (T588) and Veillonella parvula (ATCC 17745) were cultured to a late log phase in BHI (Difco), washed three times in 10 mM potassium phosphate buffer (PPB) and diluted to a suspension of 10^6 CFU/ml. In

5 polypropylene Eppendorf cups (Costar) 250 μ l of this suspension was mixed in duplicate with 250 μ l of an antimicrobial peptide solution according to the invention (the final peptide concentration was 100 μ g/ml) and incubated for half an hour at 37°C under

10 aerobic conditions. The control treatment was performed in 10 mM PPB without peptide.

After incubation the samples were centrifuged, 400 μ l of the supernatant was removed and 400 μ l PBS (9 mM sodium phosphate pH 7.0 in 150 mM NaCl), in which the

15 peptides are inactive, was added. The samples were further diluted in PBS and 50 μ l of tenfold and thousand-fold dilutions were plated out on blood agar (Difco) to perform viability counts.

The result of the tests is shown in table 2. This

20 shows that the peptides according to the invention have a clearly higher activity have than the naturally occurring histatin 5.

Table 1

25	Peptide	Sequence
	1	DSHAKRHHGYKRKFHEKHHSHRGY
	2	KRKFHEKHHSHRGY
	3	KRLFKELKFSLRKY
	4	KRLFKELLFSLRKY
30	5	KRLFKELKKSLRKY
	6	KRLFKELLKSLRKY
	7	OOLFOELOOSLOOY
	8	OOLFOELLOSLOOY
	9	KRLFKKLFSLRKY

	10	KRL FKLL FSLRKY
	11	LLL FLLK KKKK RY
	12	KRL FKEL KSRLKY-amide
	13	KRL FKELL FSRLKY-amide
5	14	KRKFHEKHHSHRGYC-CYGRHSHHKEHFKRK
	15	YGRHSHHKEHFKRKC-CKRKFHEKHHSHRGY
	16	^α N, ^ε N- (KRKFHEKHHSHRGY) ₂ K-amide
	17	^α N, ^ε N- (KRLFKELKFSRLKY) ₂ K-amide
	18	^α N, ^ε N- (KRLFKLLKFSRLKY) ₂ K-amide

10

Table 2

% reduction in viability counts						
	bacteria	buffer ¹	histatin 5	peptide 3	peptide 10	positive control peptide
15	<u>S. mutans</u>	0.00 (22.6)	-49.5 (68.5)	>99.9*	>99.9*	>99.9
	<u>S. sanguis</u>	0.00 (49.4)	69.5 (4.5)	99.6 (0.30)*	>99.9*	>99.9*
	<u>S. salivarius</u>	0.00 (36.3)	38.3 (44.1)	>99.9*	>99.9*	>99.9*
	<u>A. naeslundii</u>	0.00 (23.7)	-11.6 (3.9)	>99.9*	>99.9*	>99.9*
	<u>V. parvula</u>	0.00 (16.7)	26.9 (49.3)	>99.9*	>99.9*	>99.9*
	<u>F. nucleatum</u>	0.00 (15.9)	49.3 (4.5)*	92.9 (3.00)*	99.2 (0.84)*	>99.9*
20	<u>P. intermedia</u>	0.00 (4.69)	-235 (196)	-57.8 (77.9)	81.0 (21.4)*	98.0 (1.0)*

¹ The treatment with buffer was normalized at 0.00% killing. The standard deviation from the average is shown in brackets,

25 * significantly higher killing than the samples which were only administered buffer

EXAMPLE 3

30 Growth inhibition

The growth of the yeast Candida albicans, Torulopsis glabrata and meticillin-resistant

Staphylococcus aureus (MRSA) was tested by growing them on agar on which 10 µg of each of the peptides according to the invention was spotted. Table 3 shows the result. "+" designates full growth inhibition, "+/-" designates partial inhibition and "-" signifies no inhibition.

Table 3

	peptide	<u>C. albicans</u>	<u>T. glabrata</u>	MRSA
	histatin 5	-	-	-
10	dh-5	-	-	-
	peptide 3	+	+	+/-
	peptide 4	+	+	+/-
	peptide 5	+	+	n.d.*
	peptide 6	+	+	n.d.
15	peptide 7	+	+	n.d.
	peptide 8	+	+	n.d.
	peptide 9	+	+	+
	peptide 10	+	+	+
	peptide 11	+	+	+
20	peptide 12	+	+	n.d.
	peptide 13	+	+	n.d.

* not done

EXAMPLE 4Inhibition of lactic acid production

All peptides according to the invention were tested for their capacity to inhibit the lactic acid production of the bacteria Streptococcus sanguis, Streptococcus mutans, Streptococcus salivarius and Lactobacillus rhamnosus. The formation of lactic acid is a measure for the metabolic activity.

For this purpose cultures of bacteria cells were incubated for 1 hour in 10 mM PPB with 0.5 glucose and different concentrations of the peptides. The formation of lactic acid was monitored by means of spectrophotometry. Table 4 shows the result. "+" designates full inhibition at 4-20 µg/ml peptide, "-" signifies no inhibition of lactic acid formation at > 100 µg/ml peptide.

Table 4

	peptide	<u>S. sanguis</u>	<u>S. mutans</u>	<u>S. salivarius</u>	<u>L. rhamnosus</u>
20	histatin 5	-	-	-	-
	dh-5	-	-	-	-
	peptide 3	+	+	+	+
	peptide 4	+	+	+	+
	peptide 5	+	+	+	+
25	peptide 6	+	+	+	+
	peptide 7	+	+	+	+
	peptide 8	+	+	+	+
	peptide 9	+	+	+	+
	peptide 10	+	+	+	+
30	peptide 11	+	+	+	+
	peptide 12	+	+	+	+
	peptide 13	+	+	+	+

EXAMPLE 5Killing of yeasts

5 * 10⁶ cells of Candida pseudotropicalis, Candida albicans 10231, Cryptococcus neoformans, Candida krusei,
 5 Candida parapsilosis, Candida glabrata and Candida albicans 32354 and an ergosterol-deficient mutant thereof were incubated for one and a half hours at 37°C in the presence of a dilution series of a peptide according to the invention or amphotericin B in 1 mM
 10 potassium phosphate buffer, pH 7.0. The viability was determined by plating out. Used as positive control was synthetic PGLa ("Protein beginning with Glycine and ending with Leucin-~~amide~~", with the amino acid sequence GMASKGAIAGKIAKVALKAL-amide). The negative control was a
 15 peptide consisting of the residues 1 to 14 of synthetic cystatin S (SSSKEENRIIPGGI). Amphotericin is a known anti-mycotic medication. There is however one Candida albicans mutant (of strain 32354), which has no ergosterol in its cell membrane and which is resistant
 20 to amphotericin B. IC₅₀ values are peptide concentrations wherein 50% of the inoculum is killed. Table 5 shows the result.

Table 5

25	IC ₅₀ values (µg/ml)				
	peptide 10	positive control	negative control	amphotericin B	
	<u>C. pseudotropicalis</u>	2	1	>67	8
	<u>C. albicans</u> 10231	1	1	>67	1
	<u>Cr. neoformans</u>	1	0.5	>67	1
30	<u>C. krusei</u>	0.3	1	>67	>70
	<u>C. parapsilosis</u>	2	1	>67	6
	<u>C. glabrata</u>	2	9	>67	2
	<u>C. albicans</u> 32354	0.5	1	n.d.	2

<u>C. albicans</u> 32354, ergosterol-deficient mutant	1	3	n.d.	>70
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EXAMPLE 6Antibacterial activity in an artificial oral biofilm

An artificial oral biofilm was made by placing hydroxyapatite discs for five days in a continuous culture system of seven oral aerobic and anaerobic types of bacteria (Streptococcus mutans, Streptococcus sanguis, Streptococcus salivarius, Actinomyces naeslundii, Veillonella parvula, Prevotella intermedia and Fusobacterium nucleatum).

The discs with the biofilm formed thereon were subsequently incubated for half an hour with different concentrations of peptide in 10 mM potassium phosphate buffer, pH 7.0. The biofilm was then sonicated from the discs, diluted in PBS and plated out on semi-selective agar plates. The total anaerobic counts were counted (Streptococci + Actinomyces) on aerobic incubated blood agar plates, the total Gram-negative counts (Veillonella, Fusobacterium and Prevotella) on vancomycin-containing plates and the total counts on the anaerobic incubated blood agar. An inactive peptide of the Von Ebner's Gland Protein (VEGh, 3-21: LLASDEEIQDVSGTWYLKA) was used as negative control.

The results are shown in table 6. Herein "*" signifies that killing is significantly higher than of the negative control (p < 0.05)

Table 6

		% reduction in viability counts			
	treatment	concentration	total aerobic average (s.d.)	total anaerobic average (s.d.)	total Gram-negative average (s.d.)
5	buffer		0.00 (70.7)	0.00 (57.2)	0.00 (50.4)
	peptide 9	10 µg/ml	-67.4 (134.4)	-66.2 (143.6)	51.0 (32.0)*
		100 µg/ml	78.7 (25.6)*	51.2 (53.9)*	30.6 (104.4)*
		250 µg/ml	64.8 (48.4)*	61.2 (49.2)*	53.3 (70.7)
		500 µg/ml	96.6 (4.0)*	93.2 (4.1)*	96.9 (0.85)*
	peptide 10	10 µg/ml	29.9 (53.4)	54.3 (15.6)*	59.3 (41.0)*
		100 µg/ml	71.4 (26.7)*	42.5 (52.3)*	47.8 (50.0)*
	negative control peptide	100 µg/ml	-96.6 (62.4)	-71.7 (40.0)	-81.5 (183.6)
	Chlorhexidine	35 ppm	39.0 (71.8)	38.4 (63.2)	68.3 (19.6)*
		200 ppm	99.9 (0.00)*	99.9 (0.00)*	99.9 (0.00)*

10

EXAMPLE 7Antimicrobial activity on oral bacteria

in order to determine whether the peptides

15 according to the invention are also active against bacteria such as are encountered in the mouth, bacteria were collected from saliva and plaque. Saliva was shaken on a vortex mixer and centrifuged. The pellet was washed with 10 mM potassium phosphate buffer and incubated for

20 half an hour at 37°C with buffer (negative control), peptide 10 (100µg/ml; invention), PGLa (100µg/ml; positive control) and chlorhexidine (50 ppm). Table 7 shows the result.

Table 7

		% reduction in viability counts		
bacteria	treatment	total aerobic average (s.d.)	total anaerobic average (s.d.)	total Gram- negative average (s.d.)
5	saliva			
	buffer	0.00 (27.7)	0.00 (37.1)	0.00 (23.3)
	peptide 10	51.2 (31.5)*	71.8 (20.8)*	92.5 (9.15)*
	positive control peptide	60.1 (30.0)*	73.2 (12.6)*	97.7 (1.52)*
	Chlorhexidine	>99.9*	>99.9*	>99.9*
	plaque			
	buffer	0.00 (63.4)	0.00 (45.7)	0.00 (90.4)
	peptide 10	66.7 (7.17)	53.6 (30.6)	96.1 (3.02)*
	positive control peptide	58.5 (18.7)	51.5 (44.9)	84.0 (11.0)*
	Chlorhexidine	99.7 (0.3)*	99.7 (0.2)*	99.7 (0.3)*
	disrupted plaque			
	buffer	0.00 (46.0)	0.00 (52.4)	0.00 (42.7)
	peptide 10	66.9 (17.3)	57.5 (38.9)*	80.8 (24.7)*
	positive control peptide	41.7 (54.4)	68.5 (31.9)*	45.9 (77.2)*
	Chlorhexidine	99.7 (0.3)*	99.0 (1.42)*	>99.9*
	cultured plaque			
	buffer	0.00 (25.7)	0.00 (61.5)	0.00 (45.7)
	peptide 10	30.7 (50.7)	84.5 (8.75)*	28.3 (46.7)
	positive control peptide	20.6 (77.4)	87.2 (9.87)*	83.7 (7.30)*
	Chlorhexidine	>99.9*	>99.9*	>99.9*

10

EXAMPLE 8Killing of bacteria by peptide 10 compared with histatin 5

15 10^6 bacteria of the types Klebsiella (ATCC 43816) and Pseudomonas aeruginosa (PA01, clinical isolate) were incubated for 1 hour at 37°C in 10 mM sodium phosphate buffer with 1% tryptic soy broth (pH 7.4) in the presence of 25 or 50 µg/ml histatin 5, 3.12, 6.25, 12.5, 20 25 or 50 µg/ml peptide 10, 10 µg/ml protegrin (positive control), 50 µg/ml peptide 4 or no peptide (both negative controls). Further included was a blank which was not incubated for an hour at 37°C ($t = 0$ min). The colony forming units of each sample were then determined

by means of plating on DST (Diagnostic Sensitivity Test).

Figure 1A shows a histogram of the number of colony forming units (CFU) of each sample with Klebsiella.

5 Figure 1B shows the results for Pseudomonas.

EXAMPLE 9

Killing of Candida albicans by peptide 10 compared with histatin 5 with different incubation times

10 10^6 Candida albicans were incubated for 1 or 3 hours at 37°C in 10 mM sodium phosphate buffer with 1% Sabouraud (pH 7.4) in the presence of 25, 50 or 100 µg/ml histatin 5, 3.12, 6.25, 12.5, 25, 50 or 100 µg/ml peptide 10, 10 µg/ml protegrin (positive control), 50
15 µg/ml peptide 4 or no peptide (both negative controls). Further included was a blank which was not incubated for one hour at 37°C (t = 0 min). The colony forming units of each sample were then determined as described above with Sabouraud plates.

20 Figure 2A shows a histogram of the number of colony forming units (CFU) of each sample after 1 hour of incubation. Figure 2B gives the results for 3 hours of incubation.

25

EXAMPLE 10

Killing of Salmonella and Yersinia species by peptide 10

Peptide 10 was added in a concentration of 50 µg/ml to a culture with 10^6 Salmonella typhimurium bacteria.
30 For control purposes a culture to which nothing was added was included as blank. The number of CFU was determined at the points in time 0, 1, 2 and 3 hours. The same was done with Yersinia enterocolitica (PYv+). From figures 3A respectively 3B can be seen that by
35 adding peptide 10 the number of bacteria fell to below the detection limit.

As can be seen from the above examples, the peptides according to the invention have a considerably higher antibacterial, anti-mycotic and antifungal activity than the naturally occurring histatin 5. Particular peptides
5 are also found to have a killing effect on micro-organisms which are resistant to antimicrobial agents used at present.

CLAIMS

1. Peptides with antimicrobial activity consisting of an amino acid chain which contains a domain of 10 to 25 amino acids, wherein the majority of the amino acids of the one half of the domain are positively charged
5 amino acids and the majority of the amino acids of the other half of the domain are uncharged amino acids.

2. Peptides as claimed in claim 1, **characterized in that** the domain forms an α -helix and at least at a majority of the positions 1, 2, 5, 6, 9 (12, 13, 16, 19,
10 20, 23 and 24) contains a positively charged amino acid, at position 8 a positive or an uncharged amino acid and at least at a majority of the positions 3, 4, 7, 10, (11, 14, 15, 17, 18, 21, 22, 25) contains an uncharged amino acid.

15 3. Peptides as claimed in claim 2, **characterized in that** the positively charged amino acids are chosen from the group consisting of ornithine (O), lysine (K), arginine (R) and histidine (H).

4. Peptides as claimed in claim 2 or 3,
20 **characterized in that** the uncharged amino acids are chosen from the group consisting of the aliphatic amino acids glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), the amino acids with a dipolar side chain methionine (M), asparagine (N), glutamine (Q),
25 serine (S), threonine (T), the amino acids with an aromatic side chain phenylalanine (F), tyrosine (Y), tryptophan (W).

5. Peptides as claimed in claims 2-4, **characterized in that** the majority of the positively charged amino
30 acids is the total number of positively charged amino acids minus 1.

6. Peptides as claimed in claims 2-5, **characterized in that** the majority of the uncharged amino acids is the total number of uncharged amino acids minus 1.

7. Peptides as claimed in claims 2-6, **characterized in that** the domain makes up the entire peptide.

8. Peptide as claimed in claims 2-7, of which the domain has the following amino acid sequence:

5 KRLFKELKFSLRKY (peptide 3).

9. Peptide as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKELLFSLRKY (peptide 4).

10. Peptide as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKELKKSLRKY (peptide 5).

11. Peptide as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKELLKSLRKY (peptide 6).

15 12. Peptide as claimed in claims 2-7, of which the domain has the following amino acid sequence:

OOLFOELOOSLOOY peptide 7).

13. Peptide as claimed in claims 2-7, of which the domain has the following amino acid sequence:

20 OOLFOELLOSLOOY (peptide 8).

14. Peptide as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKKLKFSLRKY (peptide 9).

25 15. Peptide as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKLLFSLRKY (peptide 10).

16. Peptides as claimed in claim 1, **characterized in that** the domain forms an α -helix and at least at a majority of the positions 1 to 6 (or 7 or 8 or 9 or 10 or 11 or 12) contains an uncharged amino acid and at position 7 (or 8 or 9 or 10 or 11 or 12 or 13) to 25 a positively charged amino acid.

17. Peptides as claimed in claim 1, **characterized in that** the domain forms an α -helix and at least at a majority of the positions 1 to 6 (or 7 or 8 or 9 or 10 or 11 or 12) contains a positively charged amino acid

and at position 7 (or 8 or 9 or 10 or 11 or 12 or 13) to 25 an uncharged amino acid.

18. Peptides as claimed in claim 16 or 17,
characterized in that the positively charged amino acids
5 are chosen from the group consisting of ornithine (O),
lysine (K), arginine (R) and histidine (H).

19. Peptides as claimed in claim 16, 17 or 18,
characterized in that the uncharged amino acids are
chosen from the group consisting of the aliphatic amino
10 acids glycine (G), alanine (A), valine (V), leucine (L),
isoleucine (I), the amino acids with a dipolar side
chain methionine (M), asparagine (N), glutamine (Q),
serine (S), threonine (T), the amino acids with an
aromatic side chain phenylalanine (F), tyrosine (Y),
15 tryptophan (W).

20. Peptides as claimed in claims 16-19,
characterized in that the majority of the positively
charged amino acids is the total number of positively
charged amino acids minus 1.

21. Peptides as claimed in claims 16-20,
characterized in that the majority of the uncharged
amino acids is the total number of uncharged amino acids
minus 1.

22. Peptides as claimed in claims 16-21,
25 **characterized in that** the domain makes up the entire
peptide.

23. Peptide as claimed in claims 16 and 18-22, of
which the domain has the following amino acid sequence:

LLLFLKKRKKRKY (peptide 11).

30 24. Peptide as claimed in claim 1, **characterized in**
that the domain forms a so-called β -strand and contains
a positively charged amino acid on at least a majority
of the positions 1, 3, 5, 7, 9 (11, 13, 15, 17, 19, 21,
23 and 25) and an uncharged amino acid on at least a
35 majority of the positions 2, 4, 6, 8, 10, (12, 14, 16,
18, 20, 22, 24).

25. Peptides as claimed in claim 24, **characterized in that** the positively charged amino acids are chosen from the group consisting of ornithine (O), lysine (K), arginine (R) and histidine (H).

5 26. Peptides as claimed in claim 24, **characterized in that** the uncharged amino acids are chosen from the group consisting of the aliphatic amino acids glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), the amino acids with a dipolar side chain
10 methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), the amino acids with an aromatic side chain phenylalanine (F), tyrosine (Y), tryptophan (W).

27. Peptides as claimed in claims 24-26,
15 **characterized in that** the majority of the positively charged amino acids is the total number of positively charged amino acids minus 1.

28. Peptides as claimed in claims 24-27,
characterized in that the majority of the uncharged
20 amino acids is the total number of uncharged amino acids minus 1.

29. Peptides as claimed in claims 24-28,
characterized in that the domain makes up the entire peptide.

25 30. Peptides as claimed in claims 1-29, wherein the N-terminus is amidated.

31. Peptides as claimed in claims 1-30, wherein the C-terminal carboxylic acid group is replaced by an amide, ester, ketone, aldehyde or alcohol group.

30 32. Peptides as claimed in any of the claims 1-31 for use as antibacterial agent.

33. Peptides as claimed in any of the claims 1-31 for use as antifungal agent.

34. Peptides as claimed in any of the claims 1-31
35 for use as anti-mycotic agent.

35. Use of peptides as claimed in claims 1-31 for the manufacture of a medicine for treating bacterial infections.

5 36. Use of peptides as claimed in claims 1-31 for the manufacture of a medicine for treating fungal infections.

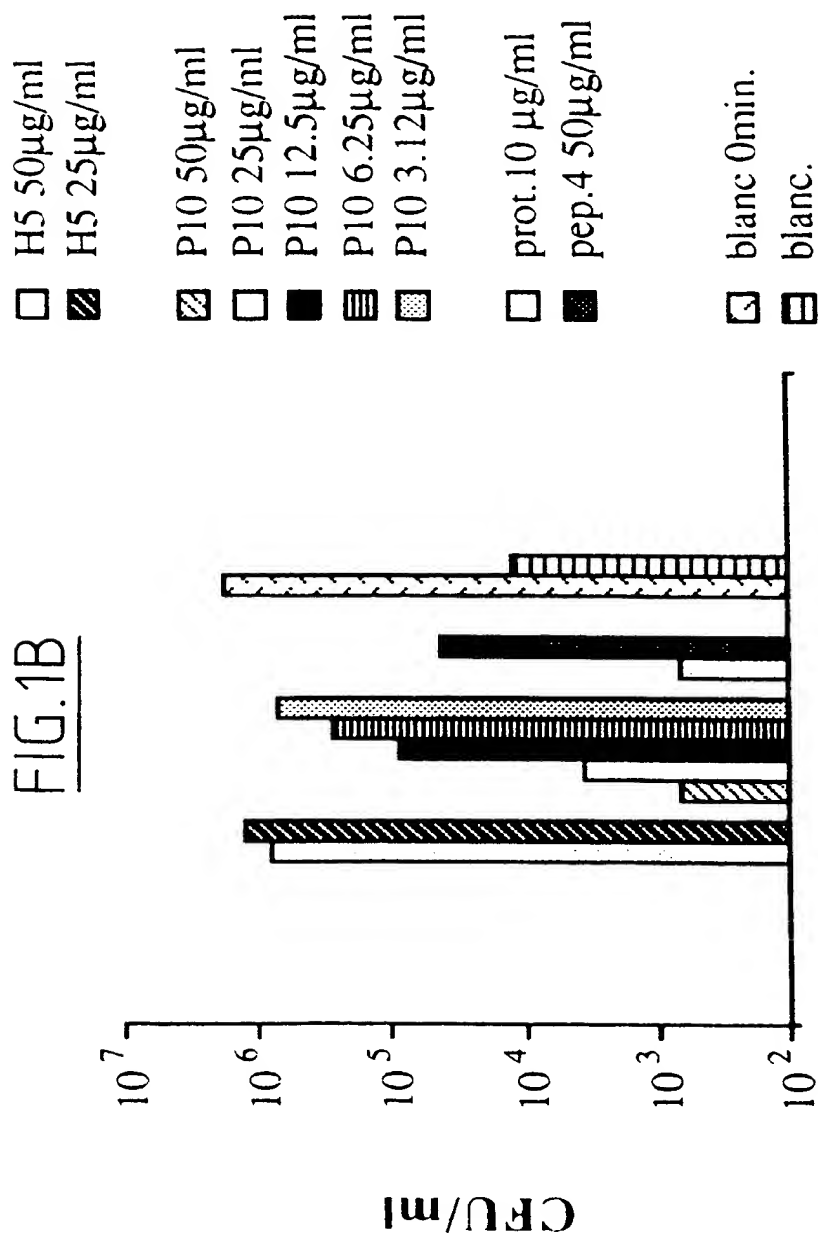
37. Use of peptides as claimed in claims 1-31 for the manufacture of a medicine for treating infections by yeasts.

10 38. Pharmaceutical composition comprising one or more peptides as claimed in claims 1-31 and one or more suitable excipients.

39. Pharmaceutical composition as claimed in claim 38 in the form of a spray, ointment, gel or lozenge.



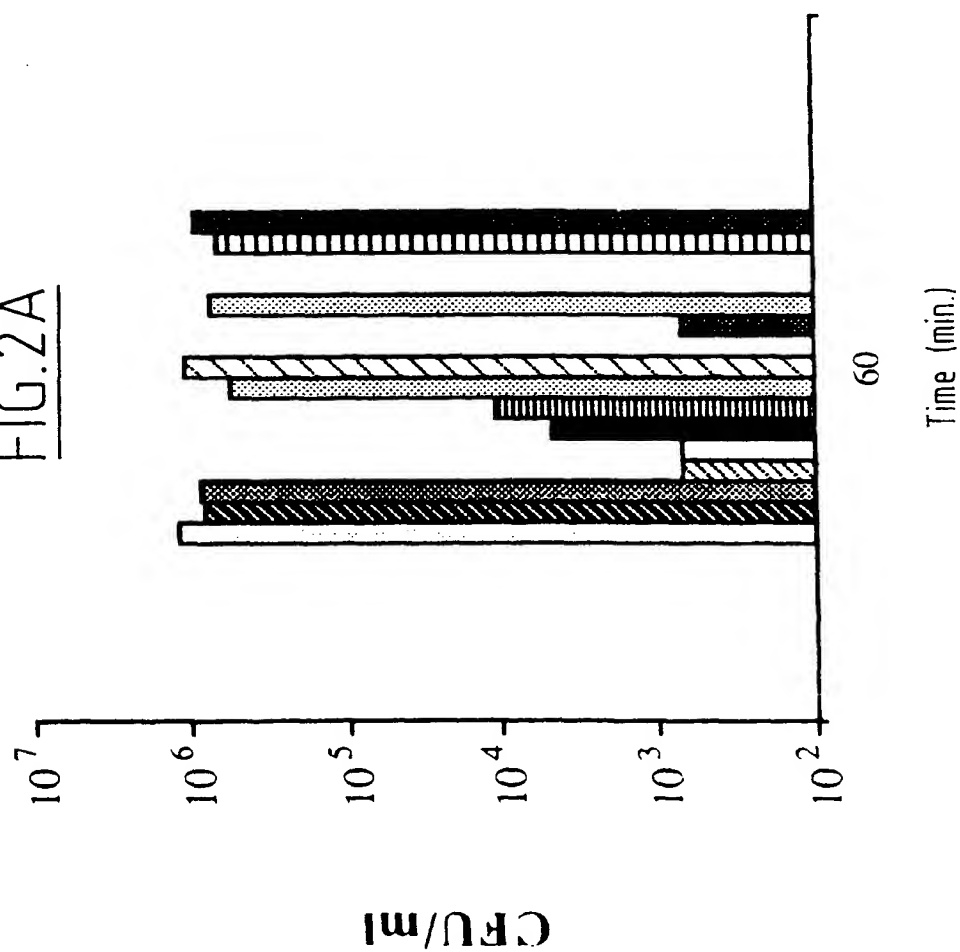
2/5

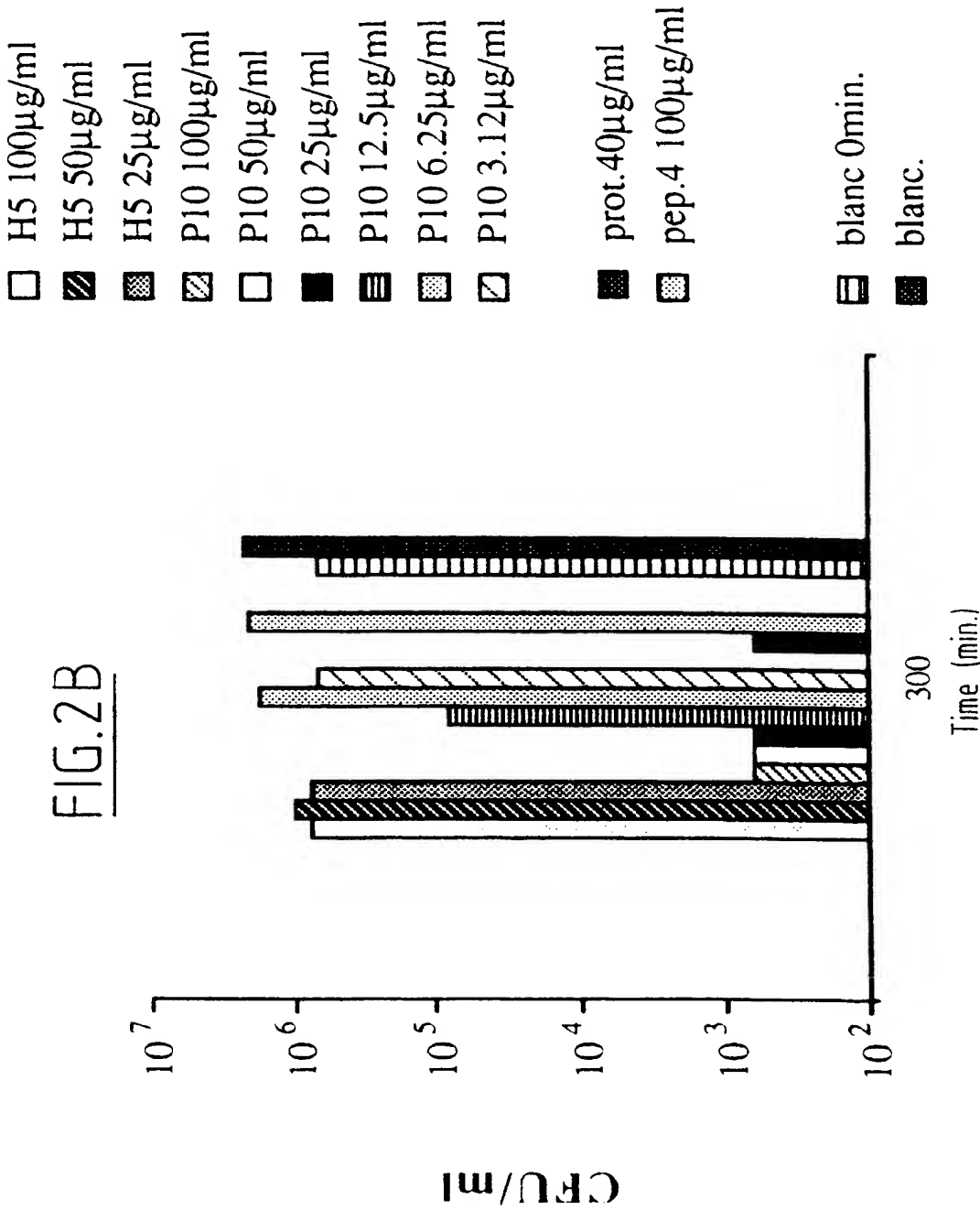


3/5

- H5 100μg/ml
 ▨ H5 50μg/ml
 ▩ H5 25μg/ml
 ▧ P10 100μg/ml
 □ P10 50μg/ml
 ■ P10 25μg/ml
 ▦ P10 12.5μg/ml
 ▤ P10 6.25μg/ml
 ▥ P10 3.12μg/ml
 ▫ prot.40μg/ml
 ▬ pep.4 100μg/ml
 ▨ blanc 0min.
 ▩ blanc.

FIG.2A





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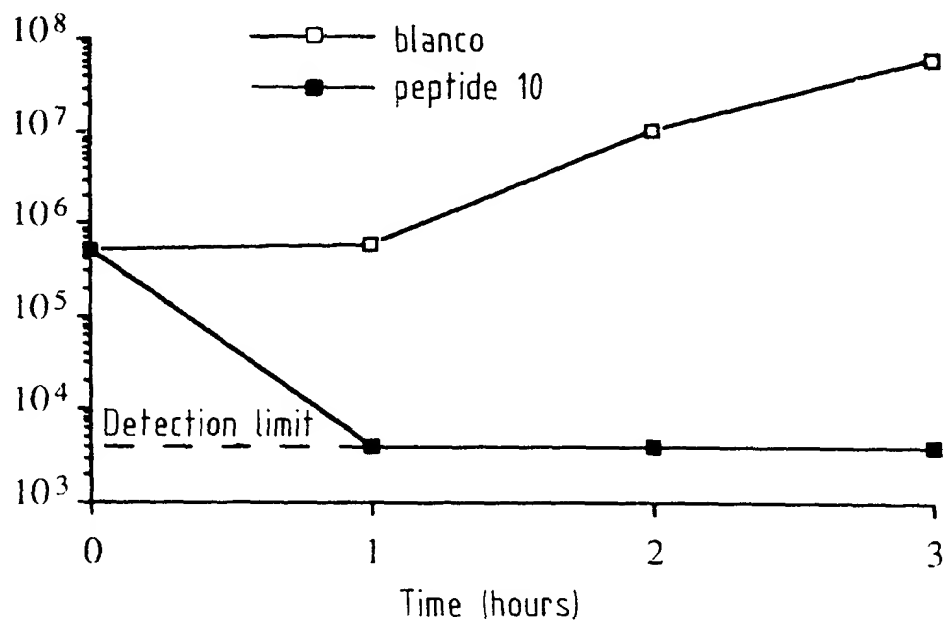
CFU *Salmonella typhimurium*

FIG. 3A

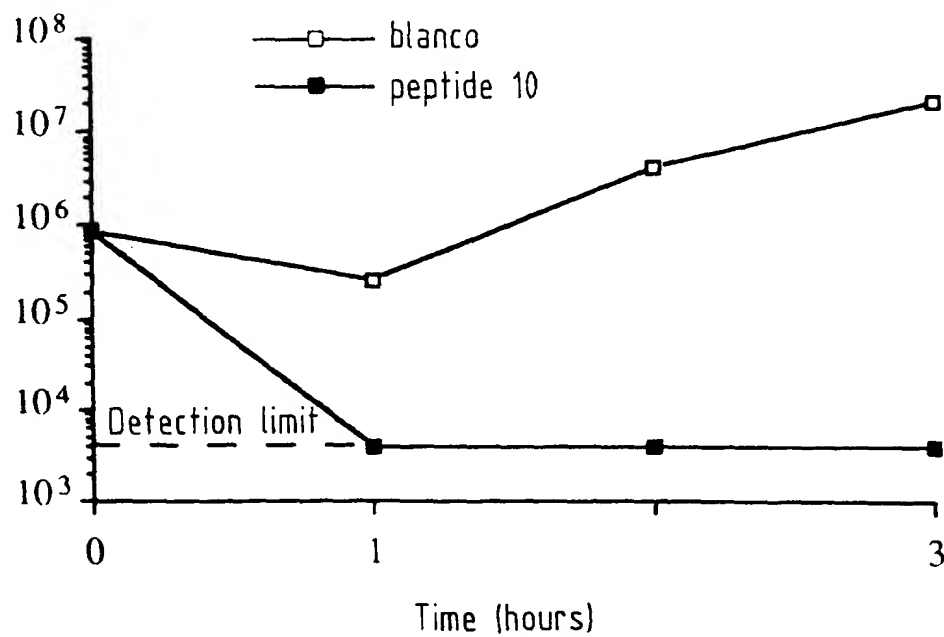
CFU *Yersinia enterocolitica* (PY^{v+})

FIG. 3B





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07K 14/47, 7/08, A61K 38/10, 38/17	A3	(11) International Publication Number: WO 99/37678 (43) International Publication Date: 29 July 1999 (29.07.99)
(21) International Application Number: PCT/NL99/00045 (22) International Filing Date: 26 January 1999 (26.01.99) (30) Priority Data: 1008139 27 January 1998 (27.01.98) NL (71) Applicant (for all designated States except US): STICHTING VOOR DE TECHNISCHE WETENSCHAPPEN [NL/NL]; Postbus 3021, NL-3502 GA Utrecht (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): VAN NIEUW AMERON- GEN, Arie [NL/NL]; Gijsbrecht van Nijenrodestraat 136, NL-3621 GM Breukelen (NL). VEERMAN, Engelmundus, Cornelis, Ignatius [NL/NL]; Willem van den Bergstraat 27, NL-1132 TZ Volendam (NL). VAN'T HOF, Willem [NL/NL]; Schelpenkade 61, NL-2313 ZZ Leiden (NL). HELMERHORST, Eva, Josephine [NL/NL]; Egelantiers- gracht 630, NL-1015 RS Amsterdam (NL). (74) Agent: VAN SOMEREN, Petronella, Francisca, Hendrika, Maria; Arnold & Siedsma, Sweelinckplein 1, NL-2517 GK The Hague (NL).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims</i> <i>and to be republished in the event of the receipt of amendments.</i> <i>In English translation (filed in Dutch).</i> (88) Date of publication of the international search report: 14 October 1999 (14.10.99)
(54) Title: ANTIMICROBIAL PEPTIDES (57) Abstract The invention relates to peptides with antimicrobial activity, consisting of an amino acid chain which contains a domain of 10 to 25 amino acids, wherein the majority of the amino acids of the one half of the domain are positively charged amino acids and the majority of the amino acids of the other half of the domain are uncharged amino acids.		

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 99/00045

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K14/47 C07K7/08 A61K38/10 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 12815 A (SQUIBB BRISTOL MYERS CO) 5 September 1991 (1991-09-05) zie Seq.ID 5-12 page 3, line 20 - page 5, line 24; claims 36,38,46; examples ---	1-6, 32-39
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X	WO 93 24138 A (MAGAININ PHARMA) 9 December 1993 (1993-12-09) zie Seq.ID's 94 ff page 5, last paragraph - page 9, paragraph 1; claims; examples ---	1-7, 32-39
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

19 August 1999

Date of mailing of the international search report

26/08/1999

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Fuhr, C

INTERNATIONAL SEARCH REPORT

Inventor's Application No
PCT/NL 99/00045

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 08270 A (MAGAININ PHARMA) 21 March 1996 (1996-03-21) zie Seq.ID 29 page 1, paragraph 4; claims; examples page 3, paragraph 4 - page 5, last paragraph ---	1-7, 32-39
X	WO 96 40251 A (OPHIDIAN PHARM INC ; WILLIAMS JAMES A (US); SHEKHANI MOHAMMED SALEH) 19 December 1996 (1996-12-19) claims; example 39 ---	1, 32-39
A	TAKAHASHI, SHO ET AL: "Synthesis of alpha.-helices having a positively charged random coil-block on either N- or C-terminal" BULL. CHEM. SOC. JPN. (1988), 61(7), 2467-71 CODEN: BCSJA8; ISSN: 0009-2673, 1988, XP002083167 page 2467, right-hand column, paragraph 2 - page 269, left-hand column, paragraph 1 ---	1, 16-22
X	TOSSI, ALESSANDRO ET AL: "Identification and characterization of a primary antibacterial domain in CAP18, a lipopolysaccharide binding protein from rabbit leukocytes" FEBS LETT. (1994), 339(1-2), 108-12 CODEN: FEBLAL; ISSN: 0014-5793, 1994, XP002083170 page 111, left-hand column, paragraph 2 - page 112, left-hand column, paragraph 3; table 1 ---	1, 24-39
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 99/00045

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 106, no. 25, 22 June 1987 (1987-06-22) Columbus, Ohio, US; abstract no. 214352, MIHARA, HISAKAZU ET AL: "Design and synthesis of basic amphiphilic peptides possessing antimicrobial activity and their interaction with lipids" XP002083172 abstract & PEPT. CHEM. (1986), VOLUME DATE 1985, 23RD, 223-8 CODEN: PECHDP;ISSN: 0388-3698, 1986, ---	1,24-39
X	CHEMICAL ABSTRACTS, vol. 103, no. 19, 11 November 1985 (1985-11-11) Columbus, Ohio, US; abstract no. 160837, SASAKI, TOORU ET AL: "Syntheses and properties of H-'Orn-Leu!n-OH (n = 1-12) and cyclo-'Orn-Leu-!n (n = 2-8)" XP002083173 abstract & PEPT. CHEM. (1985), VOLUME DATE 1984, 22ND, 361-6 CODEN: PECHDP;ISSN: 0388-3698, 1985, -----	1,24-39

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NL 99/00045

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
See FURTHER INFORMATION SHEET PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-7, 16-22 and 24-31 relate to an extremely large number of possible compounds/products. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds/products claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds/products claimed in claims 8-15 and 23. The search has been extended to closely related peptides having the alleged i.e. antibacterial activity.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/NL 99/00045

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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